

Effects of KP-496, a Novel Dual Antagonist for Leukotriene D₄ and Thromboxane A₂ Receptors, on Contractions Induced by Various Agonists in the Guinea Pig Trachea

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ABSTRACT

Background: A dry powder inhaler of KP-496 is currently in clinical development in Japan as an anti-asthmatic agent. The aim of this study was to evaluate the *in vitro* pharmacological profile of KP-496.

Methods: The antagonistic activities of KP-496 for leukotriene (LT) D₄ and thromboxane (TX) A₂ receptors were examined using the LTD₄ - and U46619-induced contractions of the isolated guinea pig trachea. The selectivity of KP-496 was examined using various agonist-induced contractions in the isolated guinea pig trachea.

Results: KP-496 produced parallel rightward shifts of the LTD₄ and U46619 concentration-response curves in a concentration-dependent manner. Schild plot analyses of the antagonistic activities of KP-496 demonstrated that it is a competitive antagonist for LTD₄ and TXA₂ receptors with pA₂ values of 8.64 and 8.23, respectively. The LTD₄ antagonistic activity of KP-496 was comparable to that of pranlukast and zafirlukast but was more potent than that of montelukast. The TXA₂ antagonistic activity of KP-496 was comparable to that of seratrodast. KP-496 and seratrodast also inhibited the prostaglandin (PG) D₂ - and PGF_{2α}-induced contractions of the isolated guinea pig trachea. KP-496 had no effect on the histamine-, acetylcholine-, serotonin- and substance P-induced contractions of the isolated guinea pig trachea.

Conclusions: These results indicate that KP-496 is a selective dual antagonist for LTD₄ and TXA₂ receptors. LTD₄ and TXA₂ play important roles in asthma, and antagonists for these mediators are being used for the treatment of asthma. Thus, KP-496 is expected to become a novel potent therapeutic agent for asthma.

KEY WORDS

antagonist, anti-asthmatic drugs, asthma, leukotriene D₄, thromboxane A₂

INTRODUCTION

Bronchial asthma is characterized by reversible airway obstruction, bronchial hyperresponsiveness and chronic inflammation. It has been reported that various chemical mediators and cytokines are involved in the onset and development of asthma. Leukotriene (LT) D₄ and thromboxane (TX) A₂ are chemical mediators and arachidonic acid metabolites.^{1,2} LTD₄ and TXA₂ induce airway smooth muscle contraction³⁻⁶ and increase the development of mucosal edema.⁷⁻¹⁰

Furthermore, LTD₄ increases mucosal secretion,^{11,12} and TXA₂ causes airway hyperresponsiveness.^{13,14} Thus, these mediators are thought to play important roles in the pathogenesis of asthma. Therefore, the regulation of their activities would provide therapeutic benefits for asthmatic patients. Currently, some potent LTD₄ receptor antagonists, such as montelukast,¹⁵ pranlukast¹⁶ and zafirlukast,¹⁷ and the TXA₂ receptor antagonist, seratrodast,¹⁸ are being used for the treatment of asthma.

LTD₄ and TXA₂ exert their biological effects via dif-

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ferent pathways. This suggests that a dual antagonist for both of these mediators may become a more potent and beneficial agent for the treatment of asthma than LTD₄ receptor antagonists and the TXA₂ receptor antagonist that have been launched. Based on this concept, KP-496, a novel dual antagonist for LTD₄ and TXA₂ receptors, was synthesized and is currently in clinical development as a dry powder inhaler. In the present study, we evaluate the *in vitro* pharmacological profile of KP-496.

METHODS

ANIMALS

Male Hartley guinea pigs (six weeks old, 350–400 g) were purchased from JAPAN SLC (Hamamatsu, Japan). The handling and treatment of the animals were in accordance with the guidelines of the Japanese Association for Laboratory Animal Science (1987).

DRUGS AND CHEMICALS

KP-496 and montelukast were synthesized by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). Pranlukast, zafirlukast and seratrodist were purified from the commercially available ONON® (Ono Pharmaceutical Co., Ltd., Osaka, Japan), ACCOLATE® (AstraZeneca, London, UK) and BRONICA® (Takeda Pharmaceutical Co., Ltd., Osaka, Japan), respectively. LTD₄, U46619, prostaglandin (PG) D₂ and PGF_{2α} were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Histamine (histamine dihydrochloride), ketotifen (ketotifen fumarate salt), serotonin, substance P, ketanserine, salbutamol and procaterol (procaterol hydrochloride) were purchased from Sigma (St. Louis, MO, USA). Carbachol (carbamylcholine chloride), indomethacin, acetylcholine (ACh) and atropine (atropine sulfate) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Salmeterol was purchased from Tocris (Bristol, UK). L-732,138 (N-Acetyl-L-tryptophan 3, 5-bis (trifluoromethyl) benzyl ester) was purchased from BIOMOL Research Lab (Plymouth Meeting, PA, USA).

KP-496, pranlukast, montelukast, zafirlukast and seratrodist were dissolved in dimethyl sulfoxide (DMSO) and diluted with Tyrode solution (136.9 mM NaCl, 2.7 mM KCl, 0.5 mM NaH₂PO₄, 1.0 mM MgCl₂ · 6H₂O, 1.6 mM CaCl₂ · 2H₂O, 5.6 mM glucose, 11.9 mM NaHCO₃). In all experiments, the final concentration of DMSO was adjusted to 0.01%.

PREPARATION OF ISOLATED GUINEA PIG TRACHEA

Guinea pigs were sacrificed by exsanguination, and the tracheae were immediately removed. Each trachea was cut into 2-mm segments in a direction parallel to the cartilage. These segments were cut open on the other side of the smooth muscle and then four sections were tied together by using a surgical adhesive. Tracheal preparations thus obtained were sus-

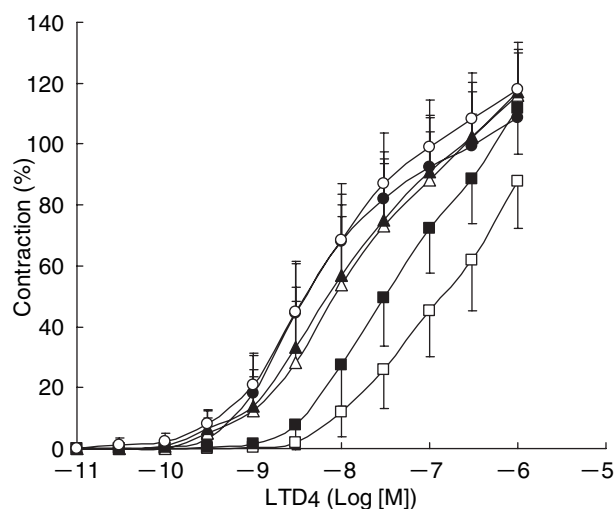


Fig. 1 Effect of KP-496 on the LTD₄ concentration-response curves of the guinea pig trachea. The results are expressed as the percentage of the response induced by 1.0×10^{-6} M carbachol and are the mean \pm SD of 6 to 34 experiments. ●, control; ○, 3×10^{-10} M KP-496; ▲, 1×10^{-9} M KP-496; △, 3×10^{-9} M KP-496; ■, 1×10^{-8} M KP-496; □, 3×10^{-8} M KP-496.

pended in Magnus tubes containing Tyrode solution. For the studies on the LTD₄-induced contractile responses, 5.0×10^{-6} M indomethacin was added to Tyrode solution to inhibit the cyclooxygenase activity. The tubes were maintained at 37°C and continuously aerated with 95% O₂ and 5% CO₂. The preparations were subjected to a 1-g load, and each preparation was equilibrated for 60 minutes. The responses of the preparations were recorded on a pen recorder (U-638, PANTOS Co., Ltd., Kyoto, Japan) via an isometric transducer (UM-203, Kishimoto Medical Instruments, Kyoto, Japan).

CONCENTRATION-RESPONSE CURVES OF LTD₄- AND U46619-INDUCED GUINEA PIG TRACHEAL CONTRACTIONS

At the beginning of the experiment, each tracheal preparation was primed using 1.0×10^{-6} M carbachol to check the contractility as well as to determine the maximum contraction of the tracheal segments. After washing with Tyrode solution and recovering to the baseline, a control cumulative concentration-response curve for LTD₄ or U46619 was obtained by increasing the concentration of either LTD₄ or U46619. After obtaining the control concentration-response curve, each preparation was equilibrated by washing with Tyrode solution and allowed to recover to the baseline. Various concentrations of the test compounds were added to the tubes and incubated for 30 minutes. Subsequently, a second concentration-response curve was obtained for the same agonist.

Table 1 Effect of KP-496 on LTD₄-induced Contractions of Guinea Pig Trachea

	Concentration (M)	n	pK _B	Schild plot analysis	
				pA ₂ (95% CI)	slope (95% CI)
KP-496	3 × 10 ⁻¹⁰	4	8.91 ± 0.50	8.64	0.98
	1 × 10 ⁻⁹	6	8.50 ± 0.40	(8.59–8.69)	(0.75–1.22)
	3 × 10 ⁻⁹	6	8.48 ± 0.70		
	1 × 10 ⁻⁸	7	8.63 ± 0.19		
	3 × 10 ⁻⁸	7	8.71 ± 0.13		

The results are expressed as the mean ± SD or the mean with 95% CI.

pA₂ and slope were calculated from a linear regression-least square method.

n: the number of experiments

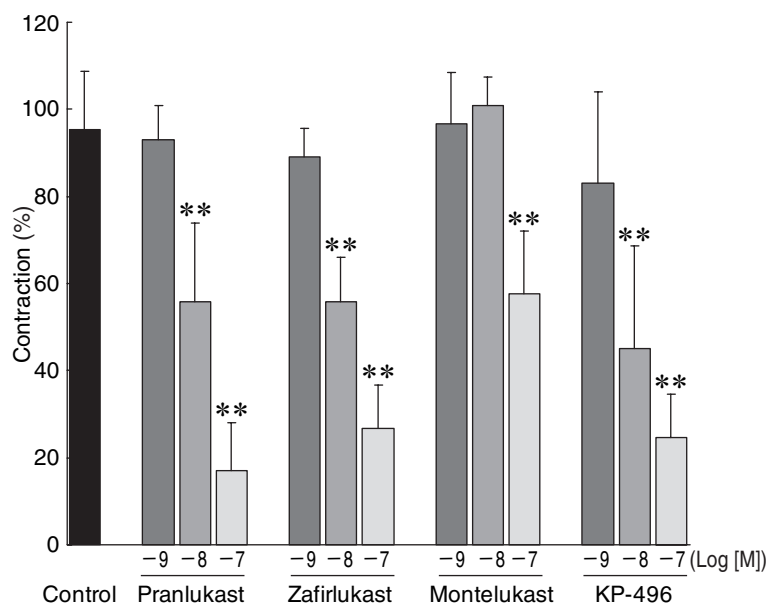


Fig. 2 Effect of KP-496, pranlukast, zafirlukast and montelukast on the LTD₄-induced guinea pig tracheal contractions. The results are expressed as the percentage of the response induced by 6.6×10^{-9} M LTD₄ and are the mean ± SD of four to six experiments. **, $p < 0.01$ compared with control (Dunnett's multiple range test).

The contractile responses were expressed as the percentage of the maximum contraction induced by 1.0×10^{-6} M carbachol. The antagonistic activity was expressed as pA₂ and pK_B. The pA₂ values were determined by Schild plot analysis,¹⁹ whereas the pK_B values were determined by Furchgott's method.²⁰ Furthermore, an estimate of the slope was also obtained, since a slope of 1 in the Schild plot analysis is indicative of a competitive antagonist.

AGONIST-INDUCED CONTRACTION OF GUINEA PIG TRACHEA

At the beginning of the experiment, each preparation was primed using the agonists (1.0×10^{-6} M carbachol, 1.0×10^{-4} M histamine, 1.0×10^{-4} M Ach,

1.0×10^{-4} M serotonin, 1.0×10^{-6} M substance P, 1.0×10^{-5} M PGD₂, 1.0×10^{-5} M PGF_{2α}) to check the contractility as well as to determine the maximum contraction of the tracheal segments. After washing with Tyrode solution and recovering to the baseline, a control contractile response was induced by each agonist (6.6×10^{-9} M LTD₄, 6.0×10^{-9} M U46619, 1.6×10^{-6} M histamine, 8.5×10^{-7} M Ach, 3.0×10^{-7} M serotonin, 1.0×10^{-7} M substance P, 3.0×10^{-7} M PGD₂, 2.0×10^{-7} M PGF_{2α}). It was confirmed that these concentrations induced stable contractions and were suitable to investigate the antagonistic activities of the test compounds in the preliminary study. After obtaining the contractile response, each preparation was equilibrated by washing with Tyrode solution and allowed

Table 2 Effect of KP-496 on U46619-induced Contractions of Guinea Pig Trachea

	Concentration (M)	n	pK _B	Schild plot analysis	
				pA ₂ (95% CI)	slope (95% CI)
KP-496	3×10^{-9}	4	8.15 ± 0.40	8.23	1.16
	1×10^{-8}	7	8.37 ± 0.58	(8.12–8.43)	(0.83–1.49)
	3×10^{-8}	6	8.20 ± 0.22		
	1×10^{-7}	6	8.49 ± 0.30		

The results are expressed as the mean \pm SD or the mean with 95% CI.

pA₂ and slope were calculated from a linear regression-least square method.

n: the number of experiments

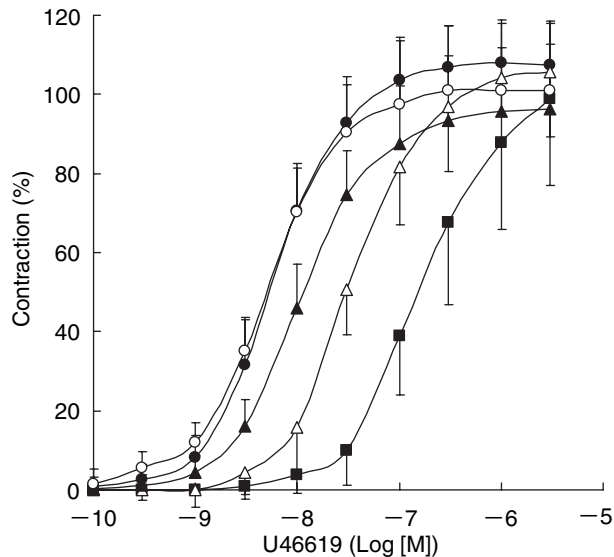


Fig. 3 Effect of KP-496 on the U46619 concentration-response curves of the guinea pig trachea. The results are expressed as the percentage of the response induced by 1.0×10^{-6} M carbachol and are the mean \pm SD of 6 to 27 experiments. ●, control; ○, 3×10^{-9} M KP-496; ▲, 1×10^{-8} M KP-496; △, 3×10^{-8} M KP-496; ■, 1×10^{-7} M KP-496.

to recover to the baseline. Various concentrations of the test compounds were added to the tubes and incubated for 30 minutes. Subsequently, a contractile response was induced by the same agonist. The contractile responses induced by LTD₄ and U46619 were expressed as the percentage of the contraction induced by 6.6×10^{-9} M LTD₄ and 6.0×10^{-9} M U46619, respectively. The contractile responses induced by the other agonists were expressed as the percentage of the maximum contraction induced by each agonist (1.0×10^{-4} M histamine, 1.0×10^{-4} M Ach, 1.0×10^{-4} M serotonin, 1.0×10^{-6} M substance P, 1.0×10^{-5} M PGD₂, 1.0×10^{-5} M PGF_{2 α}).

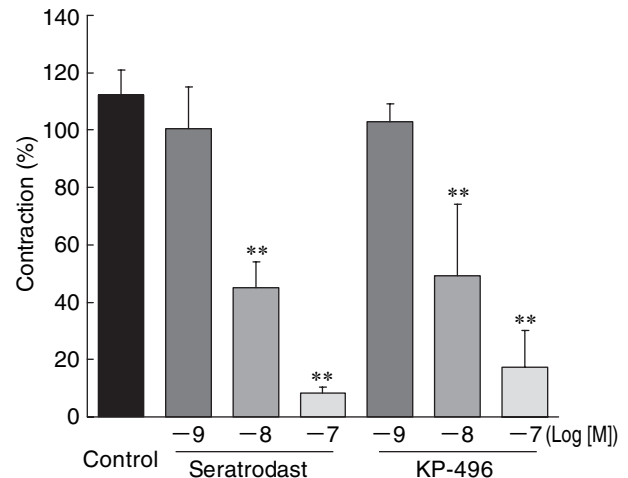


Fig. 4 Effect of KP-496 and seratrodist on the U46619-induced guinea pig tracheal contractions. The results are expressed as the percentage of the response induced by 6.0×10^{-9} M U46619 and are the mean \pm SD of four to five experiments. **, $p < 0.01$ compared with control (Dunnett's multiple range test).

β_2 -AGONIST-INDUCED RELAXATION OF GUINEA PIG TRACHEA

At the beginning of the experiment, each preparation was primed using 1.0×10^{-6} M carbachol to check the contractility as well as to determine the maximum contraction of the tracheal segments. After washing with Tyrode solution and recovering to the baseline, each preparation was primed again using 1.0×10^{-6} M carbachol. Five minutes after the contraction, a control cumulative concentration-response curve for a β_2 -agonist was obtained by increasing the concentration of the β_2 -agonist. After obtaining the first concentration-response curve, each preparation was equilibrated by washing with Tyrode solution and allowed to recover to the baseline. Various concentrations of the test compounds were added to the tubes and incubated for 5 minutes. Subsequently, a second concentration-response curve for the same β_2 -agonist

Table 3 Effect of KP-496 on Various Agonists-induced Contractions of Guinea Pig trachea

Agonist (M)	Compound	Concentration (M)	n	Contraction (%)
Histamine (1.6×10^{-6})	Control KP-496	—	6	59.17 \pm 22.17
		1×10^{-7}	5	74.07 \pm 5.85
		1×10^{-6}	5	69.32 \pm 20.46
		1×10^{-5}	5	67.36 \pm 17.48
	Ketotifen	1×10^{-7}	6	23.49 \pm 15.52**
Ach (8.5×10^{-7})	Control KP-496	—	5	81.67 \pm 10.77
		1×10^{-7}	6	84.37 \pm 6.25
		1×10^{-6}	6	77.64 \pm 6.82
		1×10^{-5}	5	90.80 \pm 11.91
	Atropine	1×10^{-7}	4	14.28 \pm 9.93**
Serotonin (3.0×10^{-7})	Control KP-496	—	6	113.18 \pm 27.03
		1×10^{-7}	6	137.25 \pm 22.14
		1×10^{-6}	6	128.61 \pm 29.73
		1×10^{-5}	6	139.26 \pm 24.13
	Ketanserin	1×10^{-6}	6	7.68 \pm 8.74**
Substance P (1.0×10^{-7})	Control KP-496	—	4	74.51 \pm 5.39
		1×10^{-7}	4	70.08 \pm 8.60
		1×10^{-6}	4	65.46 \pm 9.71
		1×10^{-5}	4	75.97 \pm 7.08
	L-732, 138	1×10^{-5}	4	42.12 \pm 10.03**
PGD ₂ (3.0×10^{-7})	Control KP-496	—	4	84.05 \pm 13.73
		1×10^{-8}	4	75.05 \pm 14.71
		1×10^{-7}	4	41.48 \pm 23.47**
		1×10^{-6}	4	26.54 \pm 6.87**
		1×10^{-5}	4	1.63 \pm 3.26**
	Seratrodast	1×10^{-7}	4	24.09 \pm 7.60**
PGF _{2α} (2.0×10^{-7})	Control KP-496	—	4	58.46 \pm 11.82
		1×10^{-8}	4	56.40 \pm 7.17
		1×10^{-7}	4	51.57 \pm 9.09
		1×10^{-6}	4	28.69 \pm 12.81**
		1×10^{-5}	4	11.25 \pm 9.76**
	Seratrodast	1×10^{-6}	4	28.16 \pm 15.97**

The results are expressed as the percentage of the maximum response induced by each agonist and are the mean \pm SD. **, $p < 0.01$ compared with control (Dunnett's multiple range test)

was obtained. The relaxation responses were expressed as the percentage of the maximum response induced by 1.0×10^{-6} M carbachol. Only one concentration-response curve was generated by salmeterol for each preparation because salmeterol caused an irreversible relaxation, and the curves obtained after the first and second cumulative addition of salmeterol were not the same.

DATA ANALYSIS

All data are represented as the mean \pm SD or the mean with 95% CI. Statistically significant differences were determined by Dunnett's multiple range tests. A

p value of less than 0.05 was defined as significant.

RESULTS

LTD₄-INDUCED CONTRACTION OF GUINEA PIG TRACHEA

LTD₄ induced concentration-dependent contractions of the isolated guinea pig trachea. KP-496 produced parallel rightward shifts of the LTD₄ concentration-response curves in a concentration-dependent manner (Fig. 1). The pK_B values were independent of the KP-496 concentrations (Table 1). The Schild plot analysis indicated that the slope was not significantly

Table 4 Effect of KP-496 on β_2 -agonist Concentration-response Curves of Guinea Pig Trachea

β_2 -agonist		Concentration (M)	<i>n</i>	EC ₅₀ (M)	<i>p</i> value
Salbutamol	Control	—	16	8.6×10^{-8}	
	KP-496	1×10^{-8}	4	10.9×10^{-8}	N.S.
		1×10^{-7}	4	10.9×10^{-8}	N.S.
		1×10^{-6}	4	10.4×10^{-8}	N.S.
		1×10^{-5}	4	9.6×10^{-8}	N.S.
Procaterol	Control	—	12	0.4×10^{-8}	
	KP-496	1×10^{-8}	3	0.4×10^{-8}	N.S.
		1×10^{-7}	3	0.5×10^{-8}	N.S.
		1×10^{-6}	3	0.4×10^{-8}	N.S.
		1×10^{-5}	3	0.4×10^{-8}	N.S.
Salmeterol	Control	—	7	14.0×10^{-8}	
	KP-496	1×10^{-8}	7	8.9×10^{-8}	N.S.
		1×10^{-7}	7	21.6×10^{-8}	N.S.
		1×10^{-6}	7	12.3×10^{-8}	N.S.
		1×10^{-5}	7	8.8×10^{-8}	N.S.

N.S. indicates no significant difference in EC₅₀ values between control and KP-496-treated groups (Dunnett's multiple range test).

n: the number of experiments

different from unity, and the pA₂ value of KP-496 was 8.64 (8.59–8.69, 95% CI) (Table 1).

KP-496, pranlukast and zafirlukast inhibited the contractile response of the isolated guinea pig trachea induced by LTD₄ (6.6×10^{-9} M) in a concentration-dependent manner and significantly inhibited the contractile response at 1×10^{-8} M. On the other hand, montelukast significantly inhibited the contractile response only at 1×10^{-7} M (Fig. 2).

U46619-INDUCED CONTRACTION OF GUINEA PIG TRACHEA

U46619 induced concentration-dependent contractions of the isolated guinea pig trachea. KP-496 produced parallel rightward shifts of the U46619 concentration-response curves in a concentration-dependent manner (Fig. 3). The pK_B values were independent of the KP-496 concentrations (Table 2). The Schild plot analysis indicated that the slope was not significantly different from unity, and the pA₂ value of KP-496 was 8.23 (8.12–8.43, 95% CI) (Table 2).

KP-496 and seratrodist inhibited the contractile response of the isolated guinea pig trachea induced by U46619 (6.0×10^{-9} M) in a concentration-dependent manner, and significantly inhibited the contractile response at 1×10^{-8} M (Fig. 4).

SELECTIVITY OF KP-496

The contractile responses induced by histamine, Ach, serotonin and substance P were significantly inhibited by the respective receptor antagonists (Table 3).

KP-496 (1×10^{-7} to 1×10^{-5} M) had no effect on

these responses. On the other hand, KP-496 significantly inhibited the contractile responses of the isolated guinea pig trachea induced by PGD₂ and PGF_{2 α} in a concentration-dependent manner (Table 3).

Salbutamol, procaterol and salmeterol increased the relaxation responses of the isolated guinea pig trachea in a concentration-dependent manner. KP-496 (1×10^{-8} to 1×10^{-5} M) had no effect on the relaxation responses induced by salbutamol, procaterol and salmeterol (Table 4).

DISCUSSION

In the present study, the pharmacological profile of KP-496 was investigated using the isolated guinea pig trachea. KP-496 produced parallel rightward shifts of the LTD₄ and U46619 concentration-response curves in a concentration-dependent manner. Schild plot analyses of the LTD₄ and U46619 antagonistic activity of KP-496 demonstrated that the slopes were not significantly different from unity. Therefore, KP-496 is a competitive dual antagonist for LTD₄ and TXA₂ receptors.

At 1×10^{-8} M, KP-496, pranlukast and zafirlukast significantly inhibited the LTD₄-induced contraction. However, montelukast significantly inhibited the LTD₄-induced contraction at a concentration of 1×10^{-7} M. These results indicate that the antagonistic activity of KP-496 is comparable to that of pranlukast and zafirlukast and is more potent than that of montelukast. This is in agreement with the previous report which showed that the antagonistic activity of pranlukast and zafirlukast is approximately ten times more potent than that of montelukast.²¹ At 1×10^{-8} M,

KP-496 and seratrodist significantly inhibited the U46619-induced contraction. Therefore, the antagonistic activity of KP-496 is comparable to that of seratrodist. LTD₄ receptor antagonists and the TXA₂ receptor antagonist are used for the treatment of asthma. These antagonists improve lung function and reduce asthmatic symptoms and the use of bronchodilators. In the present study, the antagonistic activities of KP-496 were not less potent than the activities of these antagonists; this indicates that KP-496 would also show such therapeutic effects through its antagonistic activities for LTD₄ and TXA₂ receptors.

It has been reported that PGD₂ and PGF_{2 α} are bronchoconstrictors and that their activities are exerted via their own receptors (DP and FP, respectively) and the TXA₂ receptor.²²⁻²⁴ KP-496 inhibited the contractions induced by PGD₂ and PGF_{2 α} in a concentration-dependent manner. Seratrodist also inhibited these contractions. In the preliminary study, the FP antagonist did not inhibit the contraction induced by PGF_{2 α} (data not shown). These results suggest that this *in vitro* functional assay evaluated the antagonistic activity for contractions via the TXA₂ receptor. Therefore, the antagonistic activity of KP-496 for the TXA₂ receptor is thought to contribute to these inhibitory effects on PGD₂ - and PGF_{2 α} -induced contractions.

Even at 1×10^{-5} M, KP-496 had no effect on the histamine-, Ach-, serotonin- and substance P-induced contractions, whereas at 1×10^{-8} M, KP-496 significantly inhibited LTD₄ - and U46619-induced contractions of the isolated guinea pig trachea. These results indicate that the action of KP-496 is highly selective. Further, at 1×10^{-5} M, KP-496 had no effect on the relaxations of the guinea pig trachea induced by β_2 -agonists such as salbutamol, procaterol and salmeterol. This result indicates that KP-496 does not possess bronchodilator activity and has no effect on the bronchodilator activity of β_2 -agonists. Therefore, KP-496 might not prevent the therapeutic effects of β_2 -agonists.

Recent studies demonstrated that chemical mediators such as LTD₄ and TXA₂ are involved in the pathogenesis of asthma.^{25,26} LTD₄ and TXA₂ exert their biological activities via different pathways, which suggests that inhibiting both these mediators might have an even greater therapeutic efficacy. In fact, it has been reported that the use of an LTD₄ antagonist in combination with a TXA₂ antagonist or a thromboxane synthesis inhibitor produced beneficial therapeutic effects.^{27,28} Therefore, KP-496 might become a more potent therapeutic agent for asthma than the already launched single receptor antagonists.

Although LTD₄ receptor antagonists and the TXA₂ receptor antagonist are used, there are both responders and non-responders to these antagonists. The ratio of urinary eicosanoids^{29,30} and genetic heterogeneity^{31,32} are related to the effects of these antagonists.

This suggests that the predominant mediator varies from patient to patient. KP-496 is a dual antagonist for LTD₄ and TXA₂ receptors; therefore, KP-496 is expected to be effective for a wide-range of asthmatic patients, including those who are non-responders to LTD₄ receptor antagonists or the TXA₂ receptor antagonist.

In conclusion, this study showed that KP-496 is a selective and potent dual antagonist for LTD₄ and TXA₂ receptors, and KP-496 is expected to become a useful therapeutic agent for asthma.

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